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➢ TITLE OF THE INVENTION *(280 characters max)*

AN ASSAY FOR THE DETECTION OF RAPAMYCIN AND RAPAMYCIN ANALOGS

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AN ASSAY FOR THE DETECTION OF RAPAMYCIN AND RAPAMYCIN ANALOGS

FIELD OF THE INVENTION

The present invention concerns a method for the detection of rapamycin and rapamycin analogs, as well as a kit for use in said method.

BACKGROUND OF THE INVENTION

Rapamycin (sirolimus), a macrocyclic lactone, generated by Streptomyces hygroscopicus, was initially identified 20 years ago during antibiotic screening and found to display remarkable anti fungi activity. Subsequently, rapamycin was recognized to possess highly potent immunosuppression properties and has since been used as the drug of choice in organ transplantation. More recently, the growth inhibitory effects of rapamycin have been recognized alongside the elucidation of the molecular basis of its function. The ultimate cellular target of rapamycin has been identified as a signaling kinase named *"mTOR"* that plays a central role in the control of cell proliferation and survival.

It became clear that through rapamycin binding to its intracellular receptor protein FKBP12, a complex is formed that inhibits the function of mTOR. This inhibition then results in cell cycle arrest in the G1 phase. Currently, mTOR is the only identified target of rapamycin, which places this drug in a unique position of being the most selective kinase inhibitor known. Based on these intriguing findings, the potential clinical applications of rapamycin have gone much beyond its initial development as an immunosuppressant. Rapamycin-coated cardiac arterial stents are already in clinical use to prevent intima overgrowth and the evaluation of rapamycin and rapamycin analogs as anti cancer drugs has begun. Indeed, in phase

I/II clinical trials rapamycin and its analogs have already demonstrated impressive growth-inhibitory effects against a broad range of human cancers.

Pharmacokinetic and clinical data show that rapamycin is a critical-dose drug requiring at least monthly monitoring of drug concentrations in the blood. However, current assays are based on high performance liquid chromatography coupled to mass spectrometry. These methodologies require special equipment and skills and are accordingly conducted only in specialized laboratories around the world.

As it appears that the use of rapamycin is going to increase dramatically in the near future, it would clearly be advantageous to have available a simple assay for monitoring blood levels of rapamycin and rapamycin analogs. An assay that would not require any special equipment or skills and that could be performed on a routine basis in any hospital laboratory.

SUMMARY OF THE INVENTION

The present invention provides an ELISA-based assay employing a 96-well microtiter plate to which samples, preferably blood samples, can be added, followed by a chromogenic substrate. A change in color can be monitored by a standard ELISA reader and thus translated into rapamycin concentrations.

Contrary to standard monitoring techniques for rapamycin and rapamycin analogs by HPLC and MC, the kit and method of the present invention provide a simple cheap and easily conducted assay for the determination of this important drug in a blood sample.

Thus, the present invention concerns an assay for determining rapamycin or rapamycin analog concentrations in a sample comprising:

(i) contacting the sample with PKBP12, or with a rapamycin binding fragment of PKBP12, for a time period and under conditions allowing formation of rapamycin/FKBP12 complex;

(ii) contacting the rapamycin/FKBP12 complex with a *"complex binding domain of mTOR*" for a time period and under conditions enabling binding of the complex to the *"complex binding domain of mTOR"*;

(iii) detecting the amounts of the rapamycin/FKBP12 complex bound to the *"complex binding domain of the mTOR"*;

(iv) comparing the amounts detected in (iii) above to a predetermined calibration curve, thereby determining the rapamycin concentrations in the sample.

In accordance with the present invention, the term *"rapamycin"* used hereinafter refers both to native rapamycin as generated by streptomyces hygroscopicus, as well as a synthetically produced product, as well as any analog of the two.

The term *"sample"* refers to a liquid sample, preferably a liquid sample of a body fluid such as plasma, blood, urine, sperm, cerebral spinal fluid, etc., and most preferably concerns blood.

The first step in the assay of the invention is contacting the sample with either the full FKBP12 protein being a 12kDa protein, or alternatively with a fragment of FKBP12 protein that still maintains the rapamycin binding properties of the full protein. The contact should be for a sufficient time period and under conditions which enable the formation of a rapamycin/FKBP12 complex.

Preferably, the FKBP12 protein or the fragment thereof are immobilized on a solid substrate, preferably in the form of a 96 well microtiter plate. Such immobilization enables subsequent detection of the color reaction produced by immobilized compounds.

By a preferred embodiment, the solid substrate (for example microtiter plate) is blocked by non specific protein such as BSA , which is a standard procedure used in many ELISA tests. The blocking solution is subsequentlyremoved by washing (for example by PBS) and known concentrations

of rapamycin alongside the diluted samples to be tested, preferably diluted blood samples, are added.

After step (i) of the method, a complex of rapamycin/FKBP12 complex is formed.

This complex is allowed to bind, under suitable conditions, with a *"complex binding domain of mTOR"*.

The term *"complex binding domain of mTOR"* refers to any fragment of mTOR which is known to bind the complex between the internal ligand FKBP12 and rapamycin. Typically, this is the FRB domain having 93 amino acids but may be any other fragment of the kinase that still has such a complex binding properties.

As typically the FKBP12, or the rapamycin binding fragment thereof, is immobilized onto the solid substrate (the 96 well microtiter plate) the *"complex binding domain of mTOR"* also becomes immobilized to the substrate upon its binding to the complex.

Surplus amounts of free *"complex binding domain of mTOR"* can be rinsed, and at step (iii) of the method, the detection of the amounts of *"complex binding domain of mTOR"* bound to the rapamycin/FKBP12 complex may be determined, simply by determining the amounts of immobilized *"complex binding domain of mTOR"*. The determination may be achieved by any manner known in the art, preferably by an ELISA based assay which produces a colorimetric reaction, being proportional to the amounts of *"complex binding domain of mTOR"*.

By one embodiment, the *"complex binding domain of mTOR"* such as the FRB fragment, is directly bound to a detectable label, such as bound to an enzyme capable of producing, in the presence of a suitable substrate, a color reaction. For example, the enzyme may be alkaline phosphatase which produces a color reaction in the presence of alkaline phosphate, HRP enzyme or any other enzyme known in the art to give a colorimetric reaction which may be detected by O.D reading.

By another alternative, the *"complex binding domain of mTOR"* (such as the FRB) may be detected by antibodies which they themselves are conjugated to an

enzyme capable of producing a colorimetric reaction in the presence of suitable substrate , such as alkaline phosphatase, HRP enzymes and the like.

The labeled antibodies may be directed directly against a *"complex binding domain of mTOR"* (such as the FRB domain)-i.e. elicited by immunizing an animal with FRB or an immunogenic fragment thereof, but preferably, and for ease of production, the antibodies may be directed against a tagging protein which is attached to the *"complex binding domain of mTOR"* (such as the FRB domain). The tagging protein may be any one of larger number of possible proteins such as Flag, His, HA, myc, etc. against which AP-conjugated anti epitope antibodies are available.

After step (iii) of the assay of the invention, it is possible to determine the colorimetric reaction, and the last step of the method is to attribute the color obtained as a result of step (iii) to a predefined calibration curve, so as to attribute to each O.D. reading an appropriate value of a rapamycin concentration which was previously obtained by performing the method of the invention (under the same conditions) on a plurality of samples with known and varying amounts of rapamycin or its analogs.

The present invention further concerns a kit for the determination of rapamycin concentrations, or rapamycin analog concentrations in a sample, the kit comprising:

(i) PKBP12, or a rapamycin binding portion thereof immobilized on a solid substrate;

(ii) in a separate vessel, *"a complex binding domain of mTOR"* in a form capable of being detected by a colorimetric reaction;

(iii) optionally, substrates for producing the colorimetric reaction.

(iv) optionally, pre-weighed samples of rapamycin and rapamycin analogs for producing the calibration curve.

As indicated above in connection with the method, the FKBP12, or the rapamycin binding portion thereof, can be immobilized on a 96 well microtiter

plate. Preferably, the immobilized FKBP12 is blocked with a suitable amount of an unrelated protein such as BSA.

The kit further comprises in a separate vessel a *"complex binding domain of mTOR"* preferably the 93 amino acids domain FRB, in a form capable of producing a colorimetric reaction.

By one embodiment of the kit, the *"complex binding domain of mTOR "* is conjugated directly to an enzyme capable of producing in the presence of a suitable substrate a colorimetric reaction, such as alkaline phosphatase enzyme, HRP enzyme, and the like.

By another option, the kit of the present invention contains , in an additional separate vessel, antibodies which are themselves conjugated to an enzyme capable of producing a colorimetric reaction, such as alkaline phosphatase or HRP. These antibodies should be conceptually viewed as belonging to element (ii) above as they enable the detection of the *"complex binding domain of mTOR"*.

The antibodies may be directed against the FRB fragment directly , but preferably, the FRB is conjugated to a tagging protein, and the antibodies are directed against the tagging protein as explained above in connection with the method.

Optionally, the kit further comprises the substrate required to produce the colorimetric reaction, such as in the case of alkaline phosphatase P-Nitrophenyl phosphate , Horse Radish in the case of HRP and the like.

DETAILED DESCRIPTION OF THE INVENTION

Rapamycin binds specifically and with high affinity to FKBP12, a 12kDa protein. This complex then binds to a 93 amino acids domain (FRB) present within mTOR, the cellular target of rapamycin.

A 96 well microtiter plate is coated with FKBP12 in the range 400-1000 ng/well (Protein A). The plate is then blocked by adding a solution of 2% BSA and incubating for 1h (final volume 200µl).

The blocking solution is then removed and increasing concentrations of rapamycin are added alongside diluted blood samples. Rapamycin is kept as solid and stock solutions prepared and kept for 1 month at 4°C.

By one option, after incubation, a recombinant protein comprising FRB fused to alkaline phosphatase (AP) is added (Protein B).

Following an incubation period, the wells are washed four times to remove any unbound protein.

Under these conditions, only FRB bound to the rapamycin-FKBP12 complex will remain immobilized in the wells, the amounts of the immobilized FRB bound to the rapamycin-FKBP12 complex being proportional to the rapamycin concentration.

Finally, after washing, p-Nitrophenyl phosphate (pNPP) is added and incubation continued for 20-120 min.

At the end of this incubation period, O.D. is measured by an ELISA reader at 405 nm.

The amount of pNPP hydrolyzed reflects the amount of AP in each well, which in turn is dependent on the amount of FRB retained in each well.

By another option, FRB is produced as an epitope tagged protein by cloning the PCR product into an appropriate tag carrying vector (tag options include: Flag, His, HA, myc etc.). After addition of tagged FRB to the plate, AP-conjugated anti epitope antibodies is added.

Experimental Procedures:

1. Production of the coating protein (Protein A):

A recombinant GST fusion protein comprising FKBP12, the intracellular receptor of rapamycin is generated. To this end, total RNA is isolated from the human lymphoma cell line T Jurkat that is enriched in FKBP12, by using the TRIzol™ Reagent (Life Technologies Co.). This RNA is used to synthesize cDNA using the Promega Reverse Transcription System kit (Promega, Madison, WI).

FKBP12 cDNA is then generated by PCR amplification by using the following primers:

Sense:

5'-CGCGGATCCATGGGCGTGCAGGTGGAGAC-3' and

Antisense:

5'-CGCGCTCGAGTCATTCCAGTTTTAGAAGCTCCA-3'

The PCR product is subcloned into the EcoRI/XhoI sites of the pGEX vector (Pharmacia) and transformed into TOP10 cells. Product is confirmed by DNA sequencing. The Fusion protein is induced by 1mM IPTG at 30°C for 3 h, extracted and purified on Glutathione-Sepharose beads, basically as described in (Haberman *et al.*, 2003).

2. Production of the AP-fusion protein (Protein B):

A recombinant fusion protein comprising the FKBP12 -rapamycin binding domain of mTOR is produced by PCR amplification using the cDNA described above. To this end, the following primers are used:

Sense:

5'- CGCGGATCCGAATGTGGCATGAAGGCCT-3'

Anti sense:

5'-CGCGCTCAGCTGCTTTGAGATTCGTCGGA-3'

The PCR product, which corresponds to positions 6150-6423 of mTOR is subcloned in frame to alkaline phosphatase and produced and purified from bacteria. To this end, it is possible either to use the AP-TAG kit (GenHunter) for cloning of AP fusion proteins, or the AP-fusion system (QBiogene) or to obtain the

pMY101 AP fusion vector, developed by Dr. Kay, Department of Biology, University of North Carolina, Chapel Hill.

3. Production of Epitope-tagged protein (Protein B'):

In an alternative approach, the PCR product (part 2) is subcloned into the Flag vector (Sigma) and produced as a Flag-tagged protein in bacteria. It is purified on anti Flag gel (Sigma) and added to the microtiter plate as is. At the end of the incubation period, AP-conjugated antibodies directed against the Flag tag are added and AP activity monitored as above.

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